THE PRESERVATION OF PNEUMOCOCCUS BY FREEZING AND DRYING

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Received for publication March 28, 1941

Swift (1937) has shown that when streptococci are completely dried while in the frozen state, they will survive unchanged for many years. Since pneumococci are more sensitive to chemical changes in their environment and undergo spontaneous autolysis more readily than do streptococci, it was of interest to determine how long pneumococcal cells would remain viable when kept in the desiccated state. In order to ascertain whether this method of preservation, which has proved so practical in the case of other bacteria, is equally applicable to pneumococci, the following experiments were carried out. During the winter of 1935–1936 freshly isolated strains of types I, II, III and VIII pneumococci were prepared and dried by the technic described by Swift. The viability of the bacterial cells preserved under these conditions was tested by cultural methods after a period of three years. The biochemical and immunological characteristics, as well as the virulence of the recovered cultures, were compared with those of the parent strain.

METHODS

Strains of pneumococcus of types I, II, III, and VIII freshly isolated from human sources and of known virulence were grown in flasks containing 50 ml. of blood broth for 18 hours at 37°. The cultures were centrifuged and the cells resuspended in 1 ml. of plain broth. Two-tenths ml. portions of the concentrated bacterial suspension were distributed in small glass tubes measuring 9½ x 90 mm.

A slightly modified CO_2 method of freezing the cultures was

used. Instead of placing the CO_2 ice in the glycerol, the concentrated cultures were first rapidly frozen by holding them on a cake of CO_2 ice and then placed immediately in the already chilled glycerol in a desiccating jar. A large flat dish, containing P_2O_5, was placed on top of the tubes, and the cover of the jar was replaced and pressed down to insure a tight seal. The jar was exhausted of air by means of a vacuum pump. The desiccator was then placed in the refrigerating box overnight. A refrigerator box large enough to hold the desiccating jar, such as used by Swift, is cooled by placing 200 to 400 grams of solid CO_2 in the bottom. The exact amount needed to keep the inside of the apparatus slightly below 0°C. until desiccation is complete must be learned by experience. Upon removal from the desiccator, the tubes were immediately sealed by allowing heated sealing wax to run into the space above the cotton plug. Air bubbles, entrapped in the seal, can be released by gently re-heating the wax and rotating the tubes.

After the specimens had been stored for three years, single tubes were selected at random and opened. One ml. of blood broth was then added to each specimen and the mixture was incubated at 37°C. overnight. If growth was apparent the next day, the culture was plated on fresh blood agar. If no growth was apparent, a second tube of blood broth was inoculated with the contents of the first tube.

Since the presence of the minute traces of moisture will apparently allow autolytic enzymes to act and bring about lysis of the cells, the greatest care must be taken in sealing the tubes. After the tubes have been stored for a few months, gross defects of the seal can be detected by gummy appearance of the contents. However, the physical appearance of the dry contents is not necessarily an index of the viability of the organisms. In order to determine how accurately viability could be foretold by the gross appearance of the material in the tube, a note of the general condition of a number of specimens was made before the tubes were unsealed and tested. Of 125 tubes containing dried type I organisms which appeared in good condition, only 71 or 54 per cent yielded growth when subsequently cultivated in blood

broth. Likewise, of 114 apparently well-preserved specimens of type II pneumococci, only 89 or 78 per cent showed the presence of living organisms. From this it is evident that little can be foretold by the gross appearance of the dried material.

EXPERIMENTAL

Although originally 5 dried specimens of each strain were prepared, in the course of 3 years a number of them had been used, so that at the end of this period there remained only 1 or 2 tubes of certain cultures. In the majority of instances, however, 4 or 5 specimens of each strain were still available.

In table 1 are given the results of culturing 772 specimens of the frozen and dried pneumococci. From the data presented,

TABLE 1

Incidence of recovery of cultures of pneumococci after preservation for 3 years

TYPE OF PNEUMOCOCCUS	NUMBER OF SPECIMENS	VIABLE	PER CENT VIABLE
I	298	127	42
II	191	132	69
III	168	105	62
VIII	115	83	72
Total.....	772	447	57

it is seen that 57 per cent of the specimens that had been stored for 3 years still showed the presence of viable pneumococci. The number of viable specimens varied with the different types. Of the 298 samples of dried type I pneumococci, only 42 per cent yielded growth when transplanted in suitable medium. Sixty-nine per cent of the 191 specimens of dried type II pneumococci and 62 per cent of the 168 tubes containing dried type III cells showed the presence of viable organisms. From these results it would seem that the capsular development bore no relationship to the length of time an organism would live. This is further exemplified in the case of the type VIII pneumococci which, although their capsules are smaller than those of Type III, were recovered in 72 per cent of the specimens tested.

In table 2 is shown the number of strains of pneumococci of various types which were recovered from frozen and dried preparations. From this it is seen that one or more cultures grew from only 61 per cent of the 68 strains of type I pneumococci. Forty-two of the 50 strains type II (84 per cent) and 43 out of 52 type III strains (82 per cent) were recovered. In the case of the type VIII pneumococci, of the 24 strains frozen and dried, 22, or 91 per cent, of the strains were recovered. From this it is clear that there is a difference in the average length of time for which different types of pneumococci will survive after freezing and drying.

TABLE 2

Viability of strains of pneumococci after 3 years

TYPE OF PNEUMOCOCCUS	NUMBER OF STRAINS	STRAINS RECOVERED	PER CENT
I	68	42	61.7
II	50	42	84
III	52	43	82.7
VIII	24	22	91
	194	149	76.8

As each set of tubes of the same strain was dried on the same day under identical conditions, the irregularity in the survival of pneumococci of the same strain is difficult to explain. Although all tubes were carefully sealed, it is possible that this irregularity may be due to failure of the sealing of individual tubes.

The presence of type-specific substances in these cultures was tested by agglutination in dilutions of pneumococcus sera. In no instance was any change or loss of specificity apparent. The virulence for white mice following injection was also tested. The organisms had lost none of their virulence during the 3 years in the dried state.

DISCUSSION

From these experiments it is evident that pneumococci may remain viable for as long as three years after being rapidly frozen and dried. It is essential that no moisture be permitted

to enter the tube. Tubes, which have been imperfectly sealed, may be easily detected by the gummy appearance of the material. But even tubes, the contents of which appear to be dry, fail to contain viable organisms. This is probably due to the penetration of minute amounts of moisture which allow the lytic enzymes to function. But there also may be a difference in the capacity of individual organisms from different strains to live. In the case of dried sputum (Stillman, 1938), where no attempt was made to exclude moisture, type I organisms lived on an average for 4 weeks and type III for 8 weeks.

When comparatively large quantities of organisms were dried in rabbit's blood and likewise stored without any attempt to exclude moisture (Stillman, 1940), although the period of survival was much shorter, the same tendency for the type I organisms to die most rapidly and the type III to live longest was also noted. In these frozen and dried specimens the type I cultures also apparently died more rapidly.

SUMMARY

1. Pneumococci in the dried state may remain viable for at least 3 years.

2. Variations in the viability in the different types of pneumococci have been observed under these conditions.

3. The serological specificity and virulence of strains recovered after freezing and drying remain unaltered.

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